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Control of Cell Pattern in the Neural Tube: Regulation of Cell Differentiation by *dorsalin-1*, a Novel TGFβ Family Member

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Summary

Distinct cell types differentiate along the dorsoventral axis of the neural tube. We have cloned and characterized a novel member of the TGFβ gene family, *dorsalin-1* (*dsl-1*), that appears to regulate cell differentiation within the neural tube. *dsl-1* is expressed selectively in the dorsal neural tube, and its pattern of expression appears to be restricted by early signals from the notochord. Exposure of neural plate cells to dsl-1 promotes the differentiation of cells with neural crest–like properties and inhibits the induction of motor neurons by signals from the notochord and floor plate. These findings suggest that dsl-1 regulates the differentiation of cell types along the dorsoventral axis of the neural tube, acting in conjunction with distinct ventralizing signals from the notochord and floor plate.

Introduction

Inductive interactions that define the fate of cells within the neural tube establish the initial pattern of the embryonic vertebrate nervous system. In the spinal cord, the identity of cell types is controlled, in part, by signals from two midline cell groups, the notochord and floor plate, that induce neural plate cells to differentiate into floor plate, motor neurons, and other ventral neuronal types (van Straaten et al., 1988; Smith and Schoenwolf, 1989; Placzek et al., 1990a, 1993; Yamada et al., 1991; Hatta et al., 1991). The induction of floor plate cells appears to require a contact-mediated signal (Placzek et al., 1990a, 1993), whereas motor neurons can be induced by diffusible factors (Yamada et al., 1993 [this issue of *Cell*]). Thus, the fate of different ventral cell types may be controlled by distinct signals that derive from the ventral midline of the neural tube.

The specification of dorsal cell fates appears not to require ventral midline signals since the neural tube still gives rise to dorsal cell types such as sensory relay neurons and neural crest cells after elimination of the notochord and floor plate (Yamada et al., 1991; Placzek et al., 1991; Ericson et al., 1992). Moreover, dorsal cell types are found at more ventral positions in such embryos (Yamada et al., 1991; Placzek et al., 1991), suggesting that many or all cells in neural tube have acquired dorsal characteristics. The acquisition of a dorsal fate could represent a default pathway in the differentiation of neural plate cells or a response to inductive factors that are distinct from the ventralizing signals that derive from the notochord and floor plate.

To identify signals that might regulate cell differentiation within the neural tube, we have searched for genes encoding secreted factors that are expressed in a restricted manner along the dorsoventral axis of the neural tube. We have focused on the transforming growth factor β (TGFβ) family since some of its members have been implicated in the control of cell differentiation and patterning in non-neural tissues. In frog embryos, for example, the differentiation and patterning of mesodermal cell types appear to be controlled, in part, by the action of activin-like molecules (Ruiz i Altaba and Melton, 1989; Green and Smith, 1990; Thomsen et al., 1990; Green et al., 1992). In addition, the dorsoventral patterning of cell types in Drosophila embryos is regulated by the *decapentaplegic* (*dpp*) gene (Ferguson and Anderson, 1992a, 1992b). The dpp protein is closely related to a subgroup of vertebrate TGFβ-like molecules, the bone morphogenetic proteins (BMPs) (Wozney et al., 1988), several members of which are expressed in restricted regions of the developing embryos (Jones et al., 1991).

We describe here the cloning and functional characterization of the *dorsalin-1* (*dsl-1*) gene, which encodes a novel BMP-like member of the TGFβ superfamily. *dsl-1* is expressed selectively by cells in the dorsal region of the neural tube, and its expression in ventral regions appears to be inhibited by signals from the notochord. Dsl-1 promotes the differentiation or migration of neural crest cells and can prevent the differentiation of motor neurons in neural plate explants. The combined actions of dsl-1 and ventralizing factors from the notochord and floor plate may regulate the identity of neural cell types and their position along the dorsoventral axis of the neural tube.

Results

Isolation and Characterization of *dsl-1*

To isolate novel members of the TGFβ family, we used degenerate oligonucleotides directed against conserved sequences present in the subfamily of TGFβ members that includes the BMPs, Vg-1, and dpp (Wharton et al., 1991). Oligonucleotides were used as primers in a polymerase chain reaction (PCR) to amplify sequences derived from Hamburger–Hamilton (HH) stage 16–18 (Hamburger and Hamilton, 1951) (embryonic day 2.5 [E2.5]) chick spinal cord cDNA. The PCR products were cloned, and 37 of 50 clones had inserts encoding Vg-1, dpp, and BMP-related peptides. Although most clones appeared to encode chick homologs of previously characterized *BMP* genes, one class encoded a novel sequence. A 116 bp fragment encoding this sequence was used as probe to

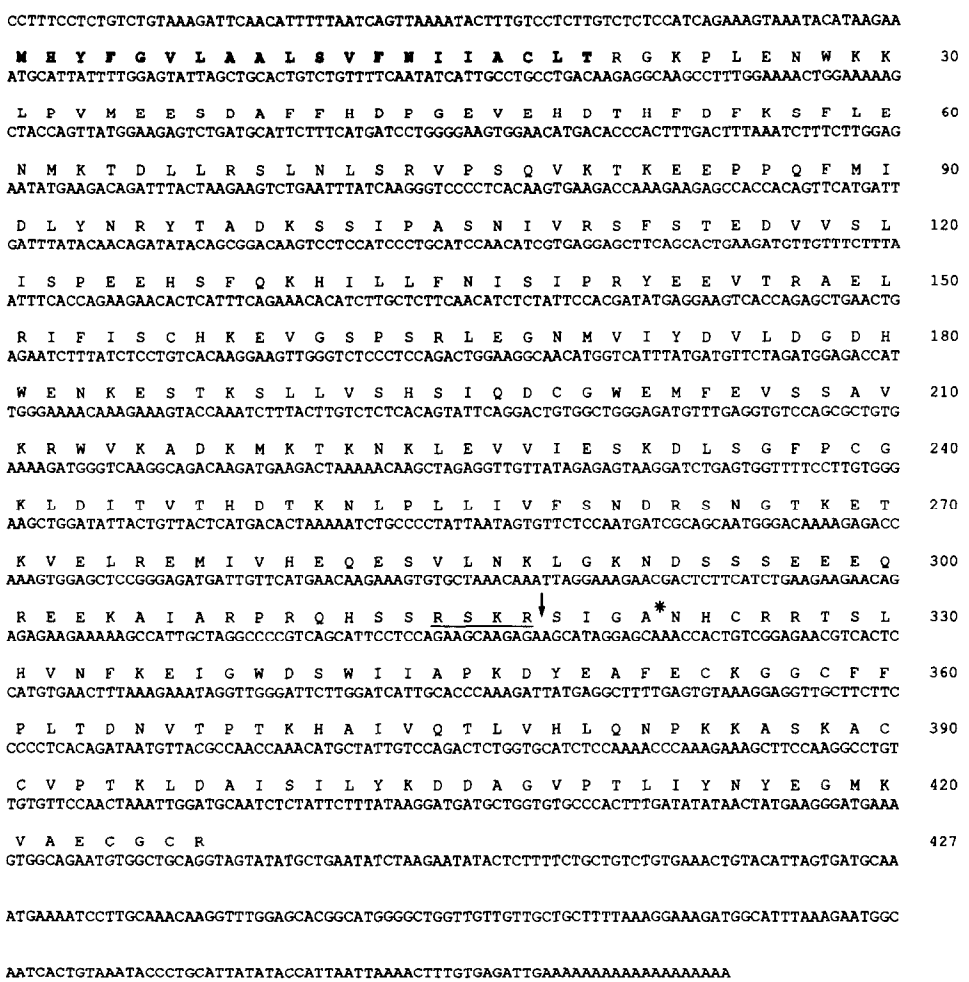


Figure 1. Nucleotide and Deduced Amino Acid Sequence of Dsl-1

The numbering of the protein sequence starts with the first methionine of the long ORF. The putative signal sequence is typed in bold letters. The RSKR sequence preceding the proteolytic cleavage site (arrow) is underlined. The site of insertion of the 10 amino acid c-Myc epitope is marked with an asterisk.

screen an E2.5 chick spinal cord cDNA library and to define a clone containing a 3.5 kb insert with an open reading frame (ORF) that encoded a protein of 427 amino acids (Figure 1).

The predicted amino acid sequence identifies this protein, dsl-1, as a novel member of the TGFβ superfamily. The N-terminal domain of dsl-1 contains a stretch of hydrophobic residues that could serve as a signal sequence. A comparison of the C-terminal 109 amino acids with those of other members of this family reveals that dsl-1 contains most of the conserved amino acids present in the other family members, including seven characteristic cysteine residues (Figure 2A). The structure of TGFβ2 (Daopin et al., 1992; Schlunegger and Grutter, 1992) suggests that intrachain disulfide bonds in dsl-1 are formed between Cys-7 and Cys-73, Cys-36 and Cys-106, and Cys-40 and Cys-108 and that Cys-72 is involved in dimer stabilization through formation of an interchain disulfide bond. The N-terminal domain of the dsl-1 precursor does not exhibit any significant similarity to other members of the TGFβ family.

Dsl-1 is more closely related to members of the Vg-1, dpp, and BMP subfamily than to the TGFβ, activin, or Müllerian inhibiting substance subfamilies (Figure 2B). Given the high degree of sequence conservation of individual members of the BMP family identified in different species (Figure 2), the divergence in sequence between dsl-1 and mammalian TGFβ family members suggests that the *dsl-1* gene encodes a novel member of this superfamily. The sequence of the mouse *dsl-1* gene (C. Cox and K. B., unpublished data) supports this idea.

As with several other family members, the conserved C-terminal region is immediately preceded by a series of basic residues that could serve as a site for proteolytic cleavage of the precursor protein (Celeste et al., 1990; Barr, 1991). To determine the site of cleavage of the dsl-1 precursor, we generated an epitope-tagged derivative, dsl-1^{Myc}, which contains a 10 amino acid insert derived from the human *c-myc* proto-oncogene (Evan et al., 1985). The c-Myc sequence was inserted two residues upstream of the first conserved cysteine (see Figure 1) in a region of the protein that exhibits no conservation with other

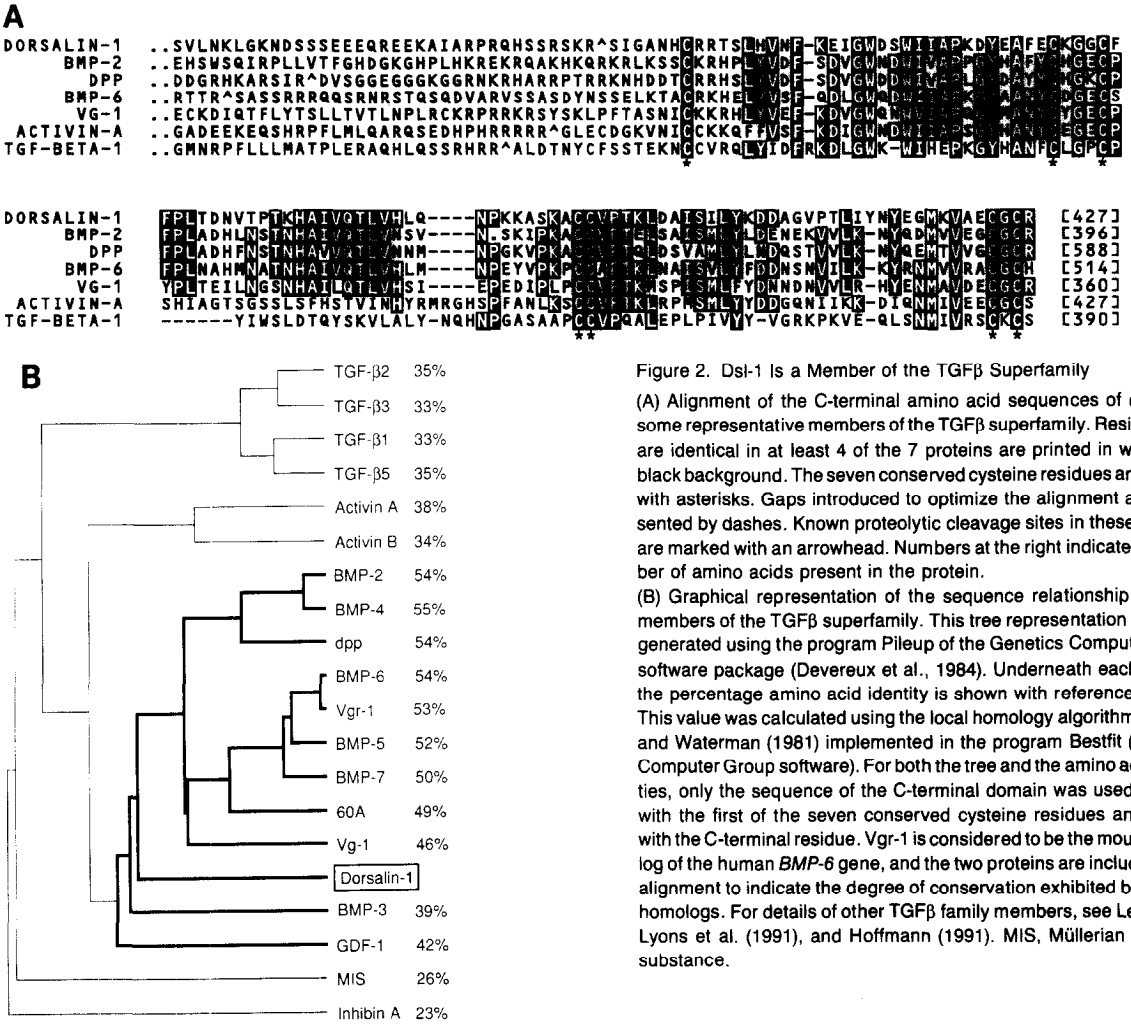


Figure 2. Dsl-1 Is a Member of the TGFβ Superfamily

(A) Alignment of the C-terminal amino acid sequences of dsl-1 and some representative members of the TGFβ superfamily. Residues that are identical in at least 4 of the 7 proteins are printed in white on a black background. The seven conserved cysteine residues are marked with asterisks. Gaps introduced to optimize the alignment are represented by dashes. Known proteolytic cleavage sites in these proteins are marked with an arrowhead. Numbers at the right indicate the number of amino acids present in the protein.

(B) Graphical representation of the sequence relationship between members of the TGFβ superfamily. This tree representation has been generated using the program Pileup of the Genetics Computer Group software package (Devereux et al., 1984). Underneath each branch, the percentage amino acid identity is shown with reference to dsl-1. This value was calculated using the local homology algorithm of Smith and Waterman (1981) implemented in the program Bestfit (Genetics Computer Group software). For both the tree and the amino acid identities, only the sequence of the C-terminal domain was used, starting with the first of the seven conserved cysteine residues and ending with the C-terminal residue. Vgr-1 is considered to be the mouse homolog of the human *BMP-6* gene, and the two proteins are included in the alignment to indicate the degree of conservation exhibited by species homologs. For details of other TGFβ family members, see Lee (1990), Lyons et al. (1991), and Hoffmann (1991). MIS, Müllerian inhibiting substance.

members of the TGFβ family (Figure 2A). cDNAs encoding native and epitope-modified dsl-1 were cloned into the expression vector pMT21 and transfected separately into COS7 cells.

Medium from cells transfected with the epitope-modified *dsl-1* construct was passed over a monoclonal antibody (MAb) 9E10 (Evan et al., 1985) anti-c-Myc affinity column. Affinity-purified proteins were analyzed by gel electrophoresis, revealing a major 15 kd band and minor bands at 45, 47, and ~60 kd (Figure 3A). The bands at 45 and 47 kd correspond in size to those predicted for the unprocessed dsl-1 protein and the 15 kd band to that expected for a proteolytically cleaved product. To establish the identity of the 15 kd band and to determine the site for proteolytic cleavage of the precursor protein, the 15 kd band was blotted onto Immobilon membranes and subjected to sequence analysis. The N-terminal sequence obtained, SIGAEQKLIS, corresponds to residues 319–322 of the predicted dsl-1 sequence, followed by the first six residues of the human c-Myc epitope. This result shows that the RSKR sequence at residues 315–318 is the site of proteolytic processing of the dsl-1 precursor (arrow in Figure 1), at least in the presence of the c-Myc peptide.

To determine whether recombinant dsl-1 secreted by COS7 cells has BMP-like activity, a biochemical assay of osteoblast differentiation was used in which BMPs induce alkaline phosphatase activity (Thies et al., 1992). Recombinant BMP-2 produced a dose-dependent increase in alkaline phosphatase activity in W-20-17 osteoblast cells over a concentration range of 10–1000 ng/μl (data not shown; Thies et al., 1992). Conditioned medium obtained from COS7 cells transfected with *dsl-1* produced an increase in alkaline phosphatase similar to that of BMP-2 at dilutions of 1:10 to 1:1000 (Figure 3B). Moreover, medium derived from COS7 cells transfected with *dsl-1*^{myc} cDNA was as effective as medium derived from cells transfected with unmodified *dsl-1* cDNA (Figure 3B). In control experiments, COS7 cells were transfected with a c-*myc*-tagged version of the Drosophila *dpp* gene, which encodes a related TGFβ family member (see Figure 2B). The dpp protein is not detected in medium conditioned by COS7 cells (K. B., unpublished data), and medium derived from *dpp* transfectants did not induce alkaline phosphatase activity, providing evidence that COS7 cells subjected to the same transfection protocol do not secrete a BMP-like activity (Figure 3B). These results show that dsl-1 can be ex-

pressed in COS7 cells in functional form, that dsl-1 mimics the activity of BMPs in this assay, and that the activity of dsl-1 is not reduced by insertion of the c-Myc peptide.

Expression of *dsl-1* mRNA in the Developing Nervous System

To examine the expression of *dsl-1* during neural development, we localized its messenger RNA (mRNA) in developing chick embryos by in situ hybridization. *dsl-1* mRNA was not expressed by cells in the neural plate (Figures 4A and 4B) and first appeared at the time of closure of the neural tube. At this stage, *dsl-1* was expressed at high levels in the dorsal third of the neural tube but was absent from more ventral regions (Figures 4C and 4D). *dsl-1* mRNA was restricted to the nervous system at this stage of development (data not shown).

The restricted expression of *dsl-1* mRNA in the spinal cord persisted after the onset of neuronal differentiation (Figures 4E–EF), and by E5, the latest stage examined, the domain of expression of *dsl-1* mRNA was confined to the dorsomedial region of the spinal cord including, but not restricted to, the roof plate (Figures 4G and 4H). *dsl-1* mRNA was also expressed in dorsal regions of the hindbrain after neural tube closure (data not shown). From E3 to E5, the only nonneural tissue types that expressed detectable levels of *dsl-1* mRNA were kidney and myotomal cells (data not shown), although the level of mRNA expression in these tissues was much lower than that in the nervous system.

Regulation of *dsl-1* mRNA Expression by the Notochord

The expression of antigenic markers that are restricted to dorsal neural tube cells is regulated by signals from the notochord and floor plate (Yamada et al., 1991; Placzek et al., 1991), raising the possibility that *dsl-1* mRNA expression is controlled in a similar manner. To examine this possibility, segments of stage 10 chick notochord were grafted into the lumen of the neural groove of host embryos prior to the onset of *dsl-1* mRNA expression. Embryos were incubated for a further 48 hr, during which time the graft was displaced dorsally such that it is eventually located at the dorsal midline of the neural tube and spinal cord. *dsl-1* mRNA expression, determined by in situ hybridization, was absent from the spinal cord of embryos with dorsal notochord grafts (Figures 5D and 5E), whereas the spinal cord of operated embryos at rostrocaudal levels that were not adjacent to the dorsal notochord graft exhibited the normal pattern of *dsl-1* mRNA expression (Figures 5A and 5B).

To correlate changes in *dsl-1* mRNA expression with neural cell pattern, sections of operated embryos adjacent to those used for in situ hybridization were examined for expression of SC1, an immunoglobulin-like protein present on floor plate cells and motor neurons (Figure 5C) (Tanaka and Obata, 1984; Yamada et al., 1991). In embryos in which *dsl-1* mRNA was absent from the spinal cord, SC1 expression revealed a duplication of ventral pattern in the dorsal spinal cord (Figure 5F). Thus, dorsal notochord grafts prevent the onset of expression of *dsl-1* mRNA and ventralize the dorsal spinal cord.

The ability of the notochord to inhibit *dsl-1* mRNA expression suggests that the notochord might normally have a role in restricting the expression of *dsl-1* within the neural

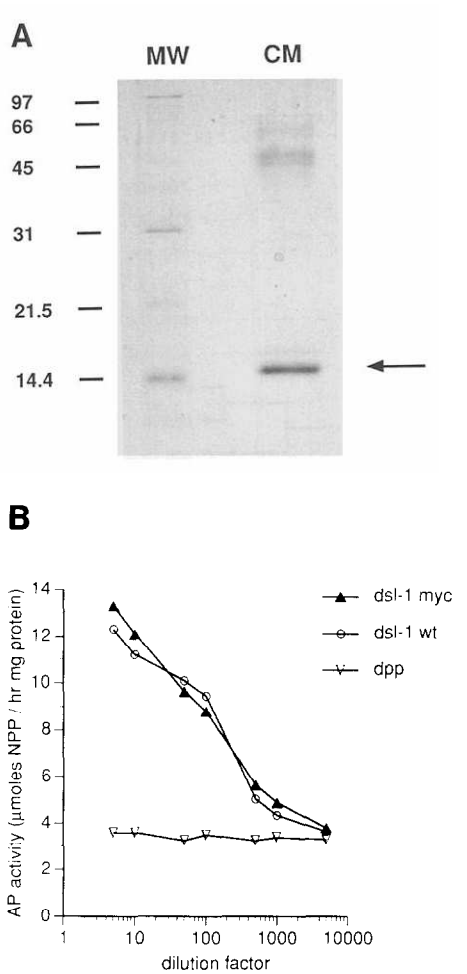


Figure 3. Affinity Purification and Functional Activity of Recombinant Dsl-1 Protein

(A) Dsl-1^{Myc} protein was purified from COS7 cell–conditioned medium using a MAb 9E10 affinity column. An aliquot of the purified protein (lane CM) was run on a 15% SDS–polyacrylamide gel and stained with Coomassie blue. The arrow points to the major product running at a molecular size of ~15 kd, and minor bands at 45, 47, and 60 kd are also evident. N-terminal sequencing of the 15 kd band confirmed its identity as processed dsl-1^{Myc} protein. Affinity-purified conditioned medium obtained from mock-transfected COS7 cells did not contain any detectable protein on a Coomassie blue–stained acrylamide gel (data not shown). The positions of molecular size standards (lane MW) are shown.

(B) Induction of alkaline phosphatase activity in W-20-17 cells by dsl-1. Conditioned medium was harvested from COS7 cells transfected with *dsl-1* and *dsl-1*^{myc} cDNAs and added at different dilutions to W-20-17 cells for 72 hr, and alkaline phosphatase activity was assayed (Thies et al., 1992). As a control for the presence of BMP-like activity in COS7 cells, medium was also obtained from cells transfected with a *c-myc*-tagged construct encoding the Drosophila *dpp* gene, a related TGFβ family member (see Figure 2B). Dpp^{Myc} is not detectable in the medium of transfected COS7 cells. Curves are from 1 of 3 experiments that produced similar results. Recombinant human BMP-2 (Thies et al., 1992) was used on a positive control in the assay.

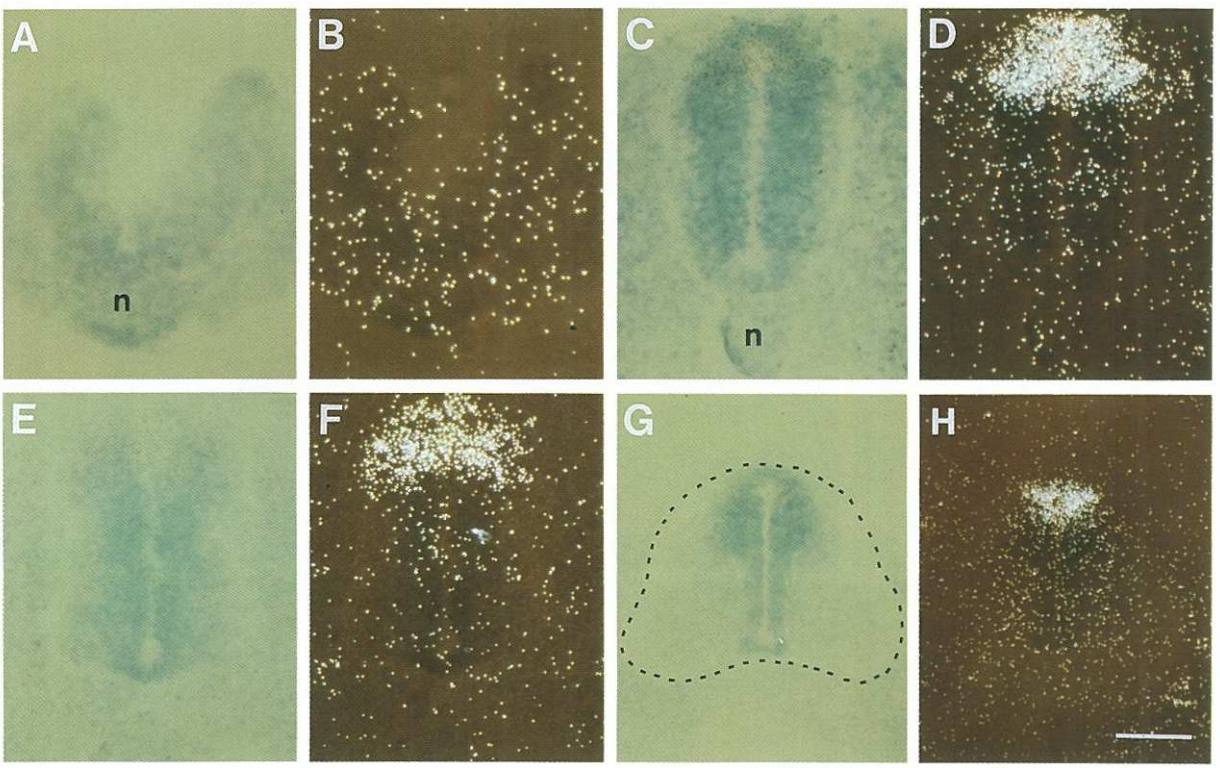


Figure 4. *dsl-1* mRNA Expression in the Embryonic Chick Spinal Cord

Panels represent pairs of phase-contrast and dark-field micrographs of sections of embryonic chick neural tube and spinal cord, processed for localization of *dsl-1* mRNA by in situ hybridization with ^{35}S-labeled probe.

(A and B) *dsl-1* mRNA is not expressed in neural cells at stages before neural tube closure. The dark-field micrograph (B) shows background grain densities.

(C and D) *dsl-1* mRNA is expressed at high levels in the dorsal third of the neural tube, beginning at the time of neural tube closure, but not by ventral neural cells or by nonneural cells. This section is taken from an HH stage 10 embryo at the future brachial level.

(E and F) The dorsal restriction of *dsl-1* mRNA persists in the spinal cord at stages after the onset of neuronal differentiation. Section taken from an HH stage 22 embryo, at the brachial level.

(G and H) At later stages of spinal cord development (HH stage 26), *dsl-1* mRNA is restricted to the dorsomedial region of the spinal cord, including but not confined to the roof plate.

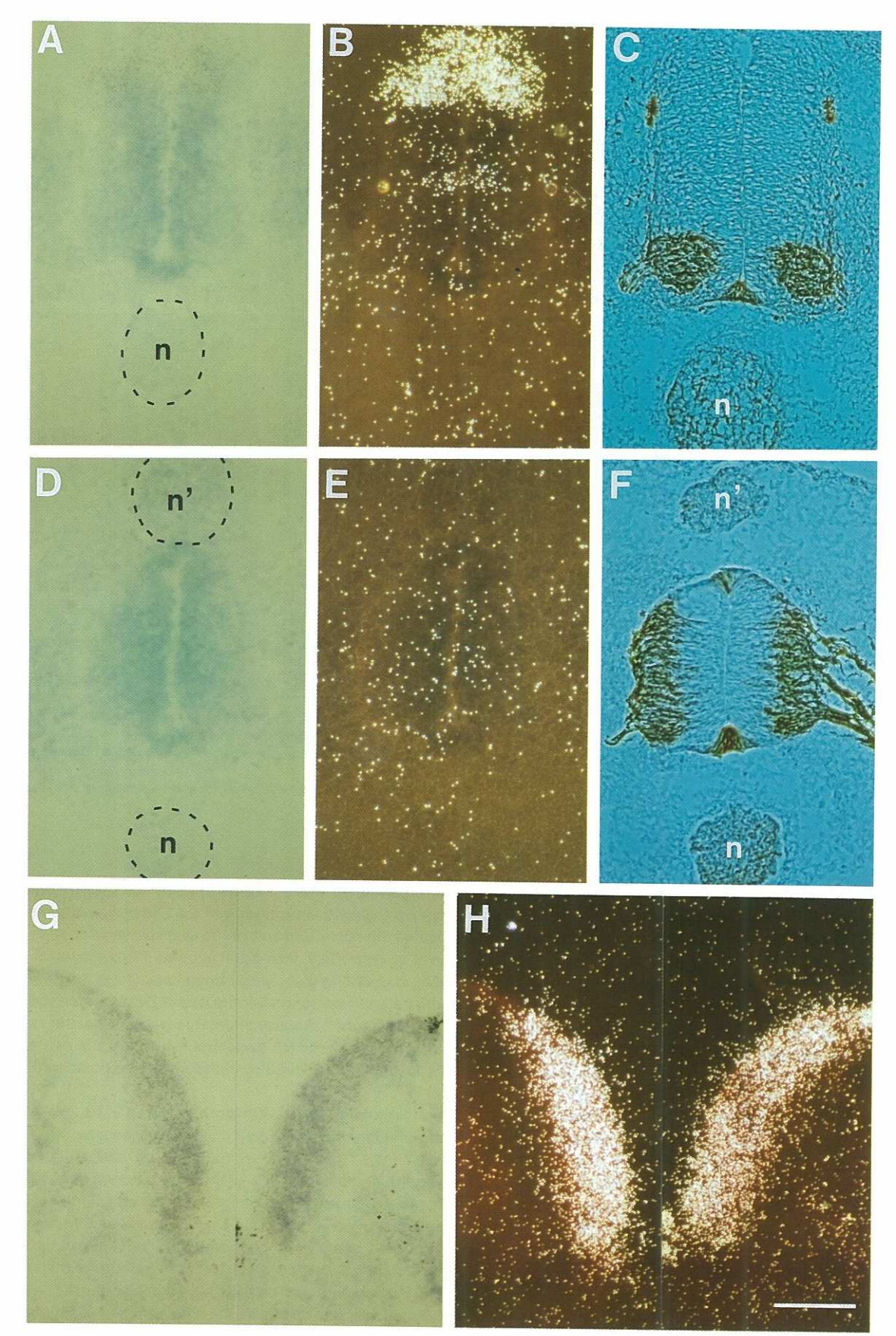
Scale bar: (A and B), 70 μm; (C–F), 80 μm; (G and H), 140 μm.

tube. Elimination of ventral midline–derived signals might therefore result in an expansion in the domain of *dsl-1* expression. To test this, Hensen's node, the precursor of the notochord, was removed from stage 10 chick embryos, thus preventing the formation of the notochord and ensuring that an early source of ventral midline–derived signals (Yamada et al., 1993) is eliminated prior to neural tube formation. The spinal cords of such embryos have been shown to lack a floor plate and ventral neurons (Grabowski, 1956; Hirano et al., 1991; Darnell et al., 1992; T. Y., unpublished data). In embryos from which Hensen's node had been removed, the domain of *dsl-1* mRNA expression expanded ventrally and, in extreme cases, included the entire dorsoventral extent of the neuroepithelium (Figures 5G and 5H). In a second series of experiments, the notochord was removed from the caudal region of stage 10 embryos, which were then permitted to develop for an additional 48 hr. At levels of the spinal cord lacking a floor plate and motor neurons, as assessed by SC1 labeling, the domain of *dsl-1* expression expanded ventrally to occupy about two-thirds of the spinal cord, although the most ventral region never expressed *dsl-1* (data not shown). The more limited ventral expansion of *dsl-1* observed after removal of the notochord compared with Hensen's node removal is consistent with other studies (Yamada et al., 1993), suggesting that ventralizing signals from the notochord begin to act soon after the neural plate has formed.

Taken together, these experiments suggest that the expression of *dsl-1* mRNA in ventral regions of the neural tube is normally inhibited by signals from the notochord.

Dsl-1 Regulates Neural Differentiation In Vitro

The dorsal restriction of *dsl-1* mRNA suggests two ways in which dsl-1 protein could regulate cell differentiation along the dorsoventral axis of the neural tube. One function of dsl-1 could be to promote the differentiation of cell types generated in the dorsal neural tube. A second function of dsl-1 could be to counteract the influence of ventralizing signals that derive from the notochord and floor plate. To begin to test the possible functions of dsl-1, we have examined its actions on the differentiation of defined cell types in neural plate explants grown in vitro. In the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following sections, we provide evidence that dsl-1 can promote the differentiation of cells with neural crest–like properties and that dsl-1 can inhibit the differentiation of motor neurons in response to inductive signals from the notochord and floor plate.

Neural Crest Cell Differentiation

Neural crest cells are generated from precursors located in the dorsal neural tube (Bronner-Fraser and Fraser, 1988). They can be identified in vitro by their ability to migrate from the neural tube; by their expression of several cell surface markers, including the HNK-1 epitope (Maxwell et al., 1988), β_1 integrin (Delannet and Duband, 1992), and the low affinity neurotrophin receptor subunit p75 (Bernd, 1985; Stemple and Anderson, 1992); and by their ability to differentiate into cell types such as neurons, glial cells, and melanocytes (Sieber-Blum and Cohen, 1980; Baroffio et al., 1988; Stocker et al., 1991).

To examine whether dsl-1 might regulate the differentiation or migration of neural crest cells, the intermediate region of the neural plate was isolated from stage 10 embryos and grown as explants in vitro (Yamada et al., 1993). As described by Yamada et al. (1993), few cells migrated from intermediate neural plate explants grown in isolation for 48 hr (Figures 6A and 6G). Addition of dsl-1^{Myc} (3×10^{-11} M) for 48 hr resulted in a 15-fold increase in the number of cells that migrated from intermediate neural plate explants (Figures 6B and 6G). To examine whether these migratory cells share surface properties with chick neural crest cells, cultures grown for 48 hr in the presence of dsl-1^{Myc} were labeled with MAbs directed against HNK-1, the β_1 integrin subunit, and chick p75. Over 90% of cells that had migrated from the intermediate neural plate explants in the presence of dsl-1^{Myc} expressed HNK-1 and β_1 integrin on their surface (Figures 6D and 6E) and approximately 30% expressed p75 (data not shown). These results show that cells induced to migrate from intermediate neural plate explants by dsl-1 have properties characteristic of neural crest cells.

To determine whether the cells that are induced to migrate from intermediate neural plate explants by dsl-1 can differentiate into cell types known to derive from the neural crest, we studied the generation of melanocytes, which can be identified unambiguously in vitro by the presence of melanin pigmentation. In these experiments we used intermediate neural plate explants from quail embryos that exhibit properties in vitro similar to those of equivalently staged intermediate neural plate explants from the nonpigmented chick strain used for all other experiments (data not shown). Melanocyte differentiation from neural crest cells in vitro has been shown to require permissive factors that can be provided in the form of chick embryo extract (CEE) or serum (Baroffio et al., 1988; Maxwell et al., 1988). Intermediate neural plate explants were therefore grown in dsl-1^{Myc} (3×10^{-11} M) for 48 hr to promote the migration of cells, after which dsl-1^{Myc} was removed and the medium supplemented with 10% CEE and 10% fetal calf serum and grown for a further 72 hr. Under these conditions, 10%–15% of the cells that had emigrated from intermediate neural plate explants expressed melanin pigment and exhibited dendritic morphology (Figure 6F), indicating the presence of melanocytes. Control experiments showed that addition of CEE and serum after exposure of intermediate neural plate explants to dsl-1^{Myc} for 48 hr did not increase further the number of migratory cells (data not shown). Moreover, melanocytes were not observed when intermediate neural plate explants were exposed to medium containing CEE and serum for 72 hr in the absence of dsl-1^{Myc} (data not shown). These results indicate that at least some of the cells induced to migrate from intermediate neural plate explants by dsl-1^{Myc} can differentiate into a defined cell type known to derive from the neural crest.

In contrast with neural crest cells that derive from the dorsal neural tube and from dorsal neural plate explants (Yamada et al., 1993), cells that had been induced to migrate from intermediate neural plate explants by dsl-1^{Myc} did not express neuronal markers or exhibit neuronal morphology when examined after 48 hr (data not shown). Taken together, these results show that dsl-1 can promote the migration of cells from neural plate explants and that dsl-1 alone does not appear to support the further differentiation of these cells into neurons. The lack of selective markers has prevented us from examining whether dsl-1 promotes the differentiation of other neural cell types that derive from the dorsal neural tube.

Figure 5. Regulation of *dsl-1* mRNA Expression by Notochord

(A and B) Phase-contrast and dark-field images of a section of spinal cord from an operated stage 22 embryo, but at a level in which there is no grafted tissue. The pattern of *dsl-1* mRNA expression is similar to that in unoperated embryos at the same developmental age.

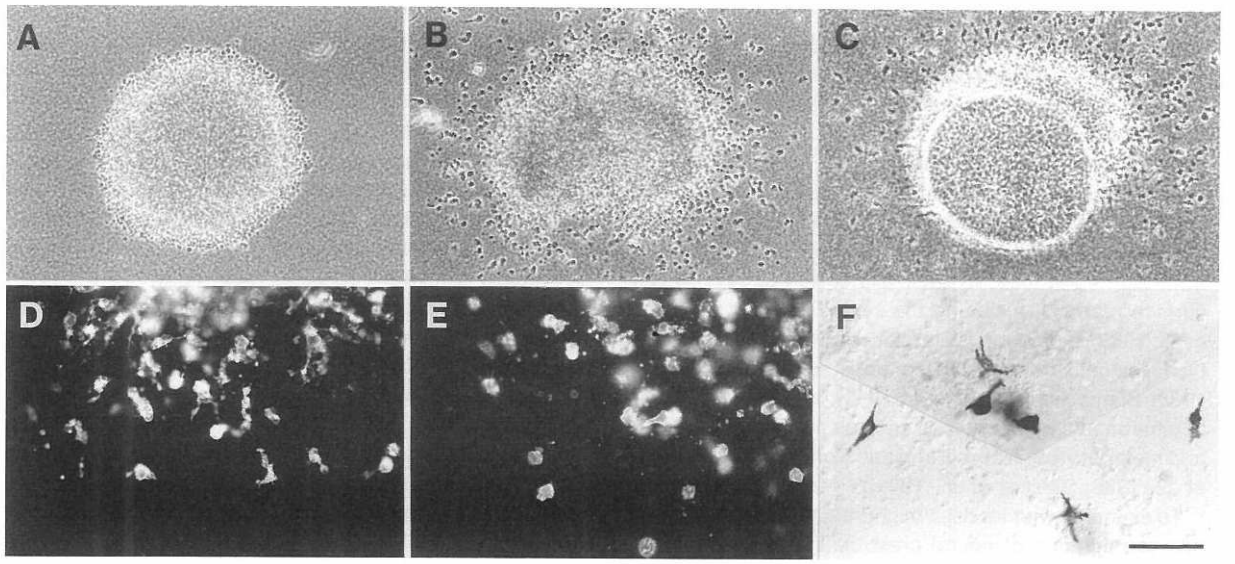
(C) Phase-contrast micrograph section from an embryo at the same stage as that shown in (A) and (B), showing the expression of SC1 by motor neurons and floor plate cells, detected by immunoperoxidase histochemistry.

(D and E) Phase-contrast and dark-field images of a section of spinal cord from an operated stage 22 embryo in which there is a dorsally located notochord (n). The expression of *dsl-1* RNA is suppressed in the presence of a dorsal notochord graft (n'). Similar results were obtained in two other embryos.

(F) Phase-contrast micrograph of an adjacent section to that shown in (D) and (E), showing the ectopic dorsal location of $SC1^+$ motor neurons that form a bilaterally symmetric continuous column. $SC1^+$ motor axons can be seen leaving the dorsal spinal cord. $SC1^+$ floor plate cells are detected at the dorsal midline. The position of the grafted notochord is indicated (n').

(G and H) Phase-contrast and dark-field micrographs showing that *dsl-1* mRNA expression expands to occupy the entire neural epithelium in embryos from which Hensen's node has been removed at HH stage 10. In this embryo the operation resulted in a splitting of the neural tube, and this micrograph has been spliced to restore the ventral apposition of neural tissue. Splitting of the neural tube occurs frequently after removal of Hensen's node (Darnell et al., 1992). A partial or complete ventral expansion of *dsl-1* expression was detected in a total of six embryos with Hensen's node removal. A ventral expression of *dsl-1* expression, occupying 60%–70% of the spinal cord, was also detected after notochord removal in two embryos.

Scale bar: (A–F), 90 μm; (G and H), 45 μm.



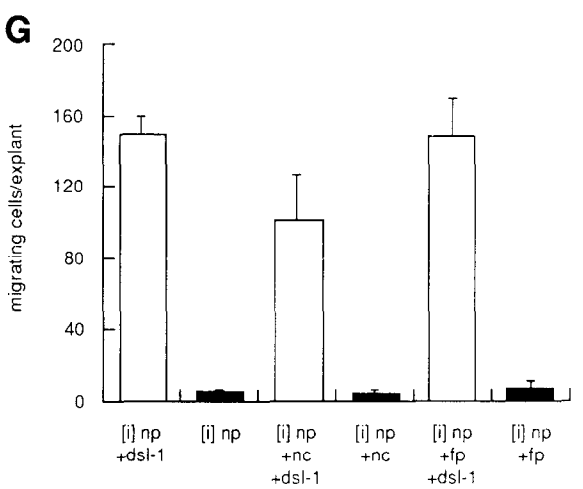


Figure 6. Induction of Cell Migration from Intermediate Neural Plate Explants by Dsl-1

Intermediate neural plate explants were grown alone or in the presence of dsl-1^{Myc} (3×10^{-11} M) for 48 hr, and migratory cells were analyzed by phase-contrast microscopy and by expression of surface antigens.

(A) Phase-contrast micrograph of intermediate neural plate explant grown alone for 48 hr.

(B) Phase-contrast micrograph of intermediate neural plate explant grown in the presence of dsl-1^{Myc} for 48 hr. Many cells have migrated from the explant.

(C) Phase-contrast micrograph of an intermediate neural plate explant grown in contact with notochord in the presence of dsl-1^{Myc} for 48 hr. Cells still emigrate from the explant, although few cells are located in the vicinity of the notochord explant.

(D) Expression of HNK-1 by cells induced to migrate from intermediate neural plate explant by dsl-1^{Myc}.

(E) Expression of β_1 integrin by cells induced to emigrate from intermediate neural plate explant. About 30% of migratory cells expressed p75, although the levels appeared lower than that detected on neural crest cells derived from the dorsal neural tube.

(F) Expression of melanin by cells induced to migrate from quail intermediate neural plate explants by dsl-1^{Myc}. In these experiments dsl-1^{Myc} was removed from after 48 hr, and cultures were grown in the presence of CEE for a further 72 hr. About 10%–15% of cells in this bright-field micrograph exhibit melanin pigment and typical dendritic morphology. Two different focal planes of the same field are shown to maintain melanocytes in focus. Similar results were obtained in six to eight explants tested. For details see text.

(G) Quantitation of cell migration induced by dsl-1. Abbreviations: [i] np, intermediate neural plate explant; nc, notochord; fp, floor plate. Error bars represent the mean ± SEM of migrated cells for 10–26 different explants.

Scale bar: (A–C), 70 μm; (D–F), 35 μm.

The presence of migratory cells was also monitored to address the fate of cells in intermediate neural plate explants that have been exposed to both ventralizing signals and to dsl-1^{Myc}. Intermediate neural plate explants grown in contact with the notochord or floor plate for 48 hr in the presence of dsl-1^{Myc} (3×10^{-11} M) exhibited a 12- to 15-fold increase in the number of migratory cells, similar to that observed when isolated intermediate neural plate explants were exposed to dsl-1^{Myc} (Figures 6C and 6G). These cells also expressed HNK-1, β_1 integrin, and p75 on their surface (data not shown). These findings show that dsl-1^{Myc} promotes the migration of cells from intermediate neural plate explants in the presence of ventralizing signals from the notochord and floor plate.

Regulation of Motor Neuron Differentiation

To examine whether dsl-1 also influences the differentiation of ventral cell types, we monitored expression of the LIM homeodomain protein Islet-1 (Karlsson et al., 1990; Ericson et al., 1992), which provides a marker for the induction of motor neurons in intermediate neural plate explants in response to diffusible signals from the notochord or floor plate (Yamada et al., 1993).

Intermediate neural plate explants grown in vitro for 48 hr contained few (usually <5) Islet-1$^+$ cells (Figures 7A and 7B; Figures 8A and 8C). In contrast, intermediate neural plate explants grown in contact with notochord or floor plate exhibited a 50- to 100-fold increase in Islet-1$^+$ cells (Figures 7D and 7E; Figure 8A). Addition of dsl-1^{Myc} to

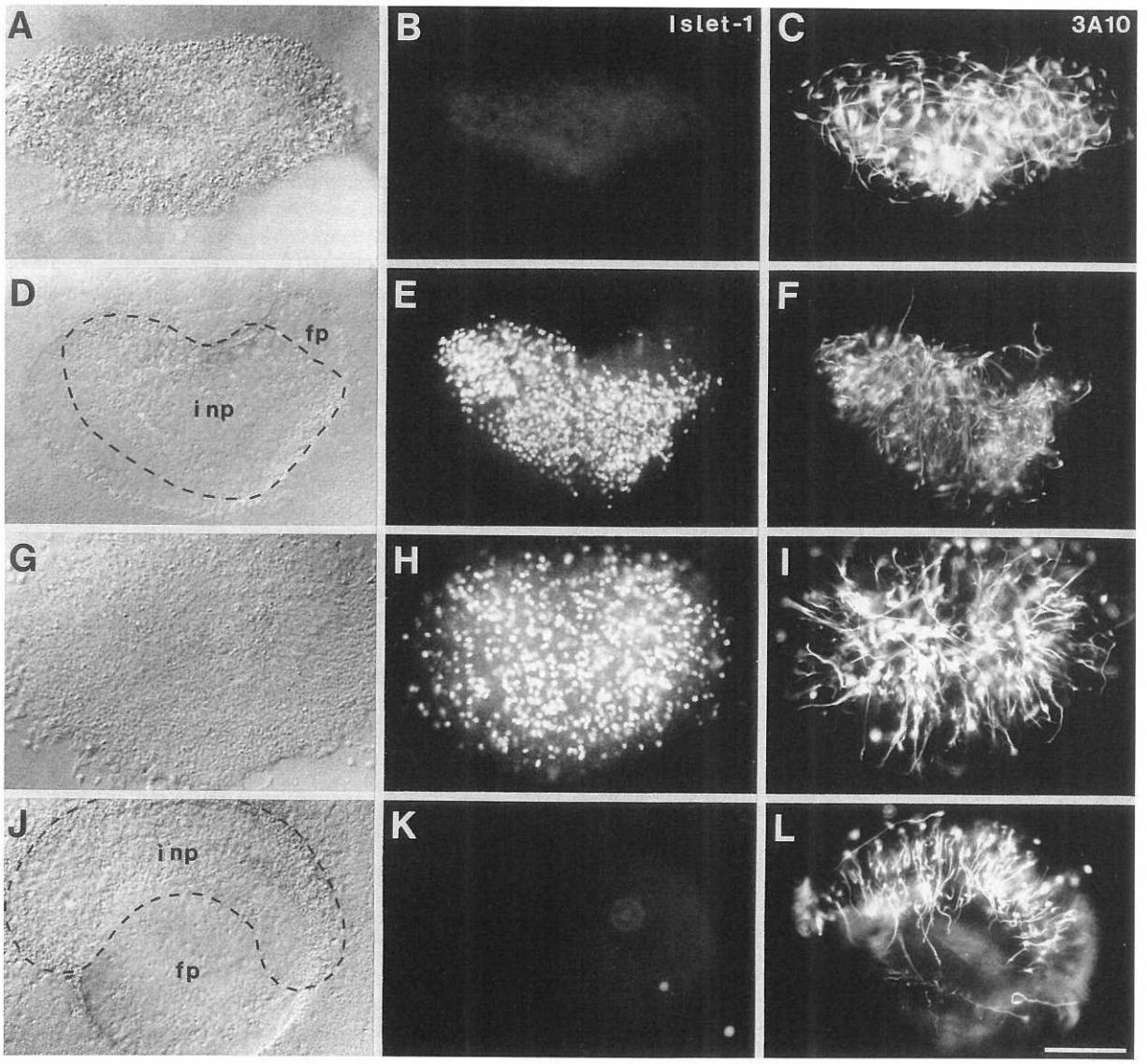


Figure 7. Induction of Islet-1 Expression in Neural Plate Explants and Suppression by Dsl-1

(A–C) Nomarski (A) and immunofluorescence (B and C) micrographs of stage 9–10 chick intermediate neural plate explant grown for 48 hr in the absence of notochord or floor plate. Islet-1$^+$ cells are not detected (B), but there is extensive neuronal differentiation as detected by 3A10 expression (C).

(D–F) Nomarski (D) and immunofluorescence (E and F) micrographs of an intermediate neural plate explant grown in contact with stage 26 chick floor plate. Numerous Islet-1$^+$ cells are present in the intermediate neural plate (i np) explant, but not in the floor plate explant (fp). The explant also contains many 3A10$^+$ cells (F).

(G–I) Nomarski (G) and immunofluorescence micrographs (H and I) of intermediate neural plate explant exposed for 48 hr to floor plate–conditioned medium. Numerous Islet-1$^+$ cells (H) and 3A10$^+$ neurons (I) are detected.

(J–L) Nomarski (J) and immunofluorescense micrographs (K and L) of an intermediate neural plate (i np) and floor plate (fp) conjugate exposed for 48 hr to 3×10^{-11} M dsl-1^{Myc}. No Islet-1$^+$ cells are detected (K), whereas the number of 3A10$^+$ neurons in the neural plate explant (L) is not obviously different from that in the absence of dsl-1^{Myc}.

In (D) and (G), the broken line outlines the extent of the neural plate (np) explant. Scale bar: (A–C), 70 μm; (D–F), 100 μm; (G–I), 70 μm; (J–L), 100 μm.

recombinates of intermediate neural plate with notochord or floor plate produced a concentration-dependent decrease in the number of Islet-1$^+$ cells present in explants (Figures 7J and 7K; Figures 8A and 8B). At concentrations of dsl-1^{Myc} of 3×10^{-11} M or greater, the differentiation of Islet-1$^+$ cells was suppressed by over 95% (Figure 8B). Dsl-1^{Myc} also abolished the expression of SC1 from regions of the intermediate neural plate explant distant from the junction with the inducing tissue (data not shown), suggesting that dsl-1^{Myc} suppresses motor neuron properties

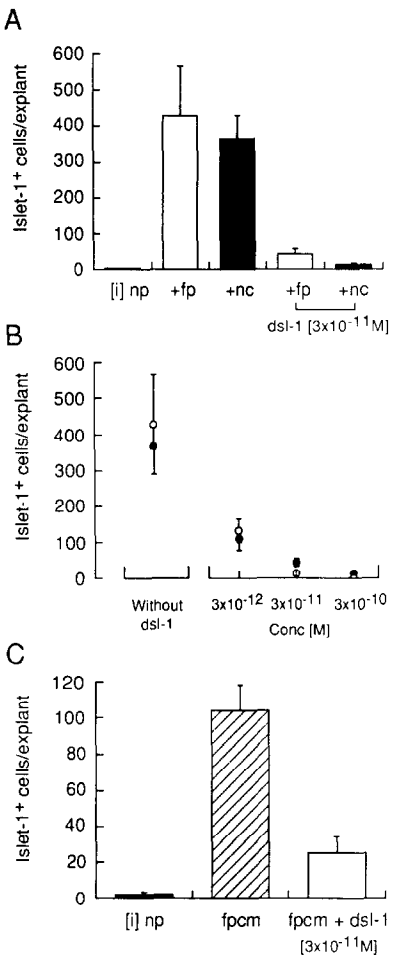


Figure 8. Inhibition of Islet-1$^+$ Cells by Dsl-1

(A) Histograms showing the induction of Islet-1$^+$ cells in intermediate neural plate ([i] np) explants by contact with notochord (nc) or floor plate (fp) and the inhibition of Islet-1$^+$ cells by dsl-1^{Myc} (3×10^{-11} M). Each column represents mean ± SEM of 10–22 different explants.

(B) Dose-dependent inhibition of Islet-1$^+$ cells by dsl-1^{Myc}. Each point represents mean ± SEM of 7–23 different explants. Open and closed circles indicate the number of Islet-1$^+$ cells induced by floor plate and notochord, respectively.

(C) Induction of Islet-1$^+$ cells by floor plate–conditioned medium (fpcm) and the inhibitory action of dsl-1^{Myc}. Each column represents mean ± SEM of 7–23 explants.

Abbreviations: [i] np, intermediate neural plate explant grown alone; +nc, neural plate–notochord conjugate; +fp, neural plate–floor plate conjugate.

other than Islet-1. Addition of dsl-1^{Myc} to neural plate explants grown alone did not induce Islet-1$^+$ cells (data not shown).

To control for the presence of COS7 cell–derived inhibitory contaminants in preparations of affinity-purified dsl-1^{Myc}, we expressed a truncated *dsl-1* cDNA in COS7 cells and compared its activity with that of native dsl-1 or dsl-1^{Myc}. The induction of Islet-1$^+$ cells by floor plate was suppressed over 95% by a 1:50 dilution of conditioned medium from COS7 cells transfected either with unmodified *dsl-1* or with *dsl-1^{myc}* cDNAs (data not shown). In contrast, medium derived from COS7 cells expressing the truncated *dsl-1* cDNA did not significantly reduce the number of Islet-1$^+$ cells induced by floor plate (364 ± 62 cells in the absence and 287 ± 45 cells in the presence of medium containing truncated *dsl-1*, mean ± SEM; n = 4, $p > 0.05$).

Dsl-1 could inhibit the generation of Islet-1$^+$ cells by preventing intermediate neural plate cells from responding to inductive signals or by inhibiting the production of this signal by the notochord and floor plate. To distinguish these possibilities, we examined the effect of dsl-1^{Myc} on Islet-1$^+$ cells in intermediate neural plate explants exposed to floor plate–conditioned medium (Yamada et al., 1993). Conditioned medium produced an ~30-fold increase in the number of Islet-1$^+$ cells (Figures 7G and 7H; Figure 8C). Addition of both dsl-1^{Myc} and floor plate–conditioned medium to neural plate explants grown alone resulted in a 76% decrease in the number of Islet-1$^+$ cells (Figure 8C). This result indicates that the inhibition of Islet-1$^+$ cells results, at least in part, from a direct action of dsl-1 on intermediate neural plate cells.

To examine whether the suppression of Islet-1$^+$ cells is accompanied by a more general inhibition of neuronal differentiation, explants processed for Islet-1 expression were double labeled with MAb 3A10, a general neuronal marker (Furley et al., 1990). Although the labeling of both cell bodies and axons by 3A10 made it difficult to count the number of neurons accurately, there was no obvious difference in the number of 3A10$^+$ cells in intermediate neural plate explants exposed to concentrations of dsl-1^{Myc}, which almost completely suppressed the differentiation of Islet-1$^+$ cells (compare Figures 7I and 7L). These results show that extensive neuronal differentiation still occurs under conditions in which the induction of Islet-1$^+$ cells is suppressed.

Discussion

Dorsoventral patterning within the neural tube appears to begin at the neural plate stage and to involve the action of both contact-mediated and diffusible inductive signals that derive initially from the notochord and later from the floor plate. A contact-mediated signal appears to be required for floor plate differentiation, whereas motor neuron differentiation can be induced by diffusible factors (Placzek et al., 1993; Yamada et al., 1993). The specification of dorsal cell types may, however, require different factors since dorsal cell types persist in the spinal cord of embryos in which the notochord and floor plate have been eliminated.

To begin to define factors involved in specifying the fate of cells in the dorsal neural tube, we cloned and characterized a novel member of the TGFβ gene family, *dsl-1*, the expression of which is restricted to the dorsal neural tube. The dorsal restriction in expression of *dsl-1* appears to be established by signals from the notochord that act on overlying neural plate cells prior to the onset of *dsl-1* transcription to prevent ventral expression of the gene after closure of the neural tube (Figure 9A). The persistence of *dsl-1* mRNA expression in the absence of the notochord and floor plate provides evidence that the expression of genes that are restricted to the dorsal neural tube is independent of ventralizing signals. Dorsal cell fates may be

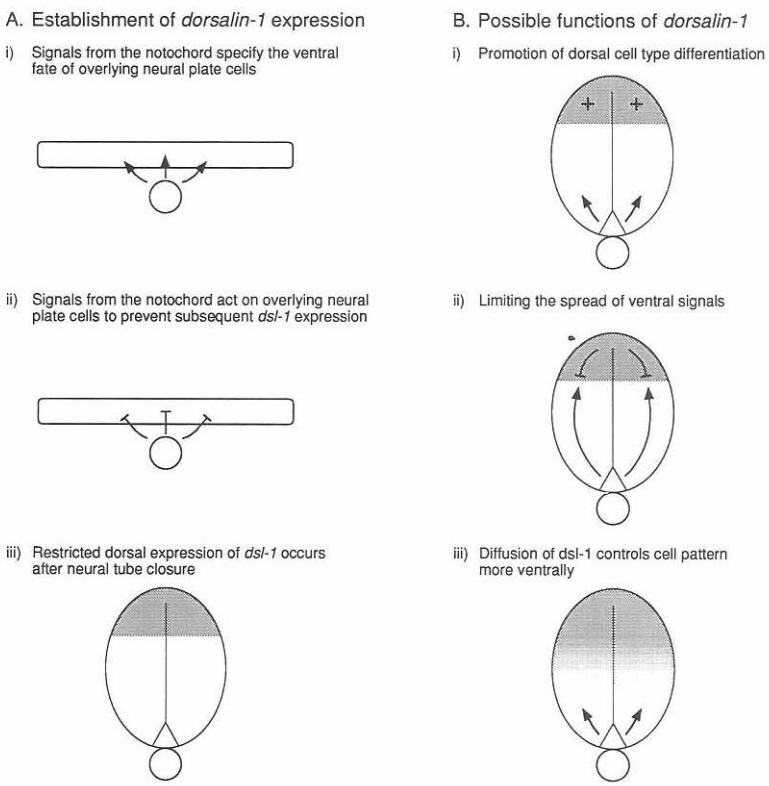


Figure 9. Potential Functions of Dsl-1 in the Control of Cell Differentiation in the Neural Tube

Diagrams summarize the possible mechanisms for establishing the dorsally restricted expression of *dsl-1* and potential functions of dsl-1 in the regulation of cell differentiation along the dorsoventral axis of the neural tube.

(A) The pattern of *dsl-1* expression appears to be established by early signals from the notochord. (Diagram i) Medial neural plate cells respond to signals from the underlying notochord that induce the differentiation of ventral cell types, such as floor plate and motor neurons. (Diagram ii) Medial neural plate cells are also exposed to signals from the notochord that prevent the subsequent expression of *dsl-1*. The inhibitory signal from the notochord can, in principle, be identical to the ventralizing signal that induces ventral cell fates. (Diagram iii) The medial region of the neural plate gives rise to the ventral neural tube. *dsl-1* expression (stippled area) begins at the time of neural tube closure and is restricted to the dorsal third of the neural tube.

(B) In vitro assays suggest several possible functions for dsl-1 in the control of neural cell differentiation. (Diagram i) Dsl-1 may promote the differentiation of cell types that derive from the dorsal region of the neural tube. In vitro studies suggest that neural crest cells represent one population of cells whose differentiation may be influenced by dsl-1. (Diagram ii) The dorsal expression of dsl-1 may define the dorsal third of the neural tube as a domain that is refractory to the long-range influence of ventralizing signals from the notochord and floor plate. The ventral boundary of *dsl-1* expression suggests that ventral midline–derived signals can influence cells over much of the dorsoventral axis of the neural tube. (Diagram iii) Dsl-1 protein may diffuse ventrally to influence the fate of cells in intermediate regions of the neural tube beyond the domain of *dsl-1* mRNA expression. Thus, the combined action of dsl-1 and the diffusible ventralizing signal from the notochord and floor plate could specify the fate of cells over the complete dorsoventral axis of the neural tube.

specified by the exposure of neural plate cells to early dorsalizing signals, perhaps from adjacent nonneural ectoderm (Takahashi et al., 1992), that induce the potential to express *dsl-1* and other dorsal genes.

Once the dorsal expression of *dsl-1* is established, dsl-1 protein could function in several different ways to control cell differentiation in the neural tube. First, dsl-1 may promote the differentiation of cell types that derive from the dorsal neural tube (Figure 9B, diagram i). Second, the expression of *dsl-1* could ensure that the dorsal neural tube is refractory to ventralizing signals from the notochord (Figure 9B, diagram ii). Finally, dsl-1 protein could diffuse and influence the fate of cells in more ventral regions of the neural tube (Figures 9A and 9B, diagrams iii). The interactions of dsl-1 and other factors from the dorsal neural tube with ventralizing signals from the ventral midline could, therefore, control the identity of cell types and the position at which they are generated along the entire dorsoventral axis of the neural tube.

Dsl-1 May Promote Neural Crest Cell Differentiation

One function of dsl-1 suggested by the pattern of expression of *dsl-1* mRNA could be to promote the differentiation of cell types that are generated in the dorsal neural tube. Neural crest cells constitute one of the major cell types that derive from precursors located in the dorsal neural tube. The present in vitro studies provide evidence that dsl-1 promotes the migration of cells with neural crest–like properties from intermediate neural plate explants. Cells exposed to dsl-1 alone appear unable to progress to fully differentiated derivatives of the neural crest; however, in the presence of additional factors, at least some migrating cells can differentiate into melanocytes. The absence of neurons or melanocytes in the population of cells induced to migrate by dsl-1 could reflect an inhibitory effect of dsl-1 on the differentiation of neural crest cells since TGFβ1 has been shown to inhibit the differentiation of neural crest cells into melanocytes (Stocker et al., 1991; Rogers et al., 1992) and to promote the production of extracellular matrix components such as fibronectin (Rogers et al., 1992) that can inhibit neuronal differentiation (Stemple and Anderson, 1992). Alternatively, other dorsally restricted factors that are absent from intermediate neural plate explants may be required for the progression of neural crest cell differentiation.

TGFβ1 has also been shown to accelerate the migration of neural crest cells from premigratory regions of the neural tube (Delannet and Duband, 1992). The action of dsl-1 to promote the migration of neural crest–like cells differs from this effect in that intermediate neural plate explants do not give rise to migratory cells in the absence of dsl-1 after longer times in vitro (Yamada et al., 1993; unpub-

lished data). Nevertheless, dsl-1 may mimic the ability of TGFβ1 to accelerate neural crest migration and could therefore be involved both in specifying the fate of premigratory neural crest precursors and in inducing the migration of these cells from the dorsal neural tube. Further studies are required to establish whether all of the cells induced to migrate by dsl-1 are neural crest cells and to define the range of cell types that can be generated from this migratory cell population. Recent studies have reported that dorsal notochord grafts do not suppress the migration of neural crest cells from chick neural tube (Artinger and Bronner-Fraser, 1992) It remains to be determined whether, under the conditions of such grafts, *dsl-1* expression is prevented.

In addition, it remains unclear whether the differentiation of other dorsal neural cell types is regulated by dsl-1. Neurons with the properties of dorsal commissural neurons can differentiate in rat neural plate explants grown in isolation (Placzek et al., 1993). Thus, it is possible that some dorsal cell types can differentiate independently of dsl-1. Alternatively, neural plate explants grown in vitro may begin to express dsl-1 at levels sufficient to drive the differentiation of some but not all dorsal cell types.

Dsl-1 as an Inhibitor of Ventral Cell Type Differentiation

Dsl-1 suppresses the differentiation of motor neurons in intermediate neural plate explants exposed to ventralizing signals from the notochord or floor plate. This finding raises the possibility that dsl-1 interacts with ventralizing signals to control cell fate along the dorsoventral axis of the neural tube. Although *dsl-1* expression detected by in situ hybridization occurs after signals from the notochord and floor plate have begun to specify ventral cell fates (Yamada et al., 1993), its expression precedes the overt differentiation of motor neurons and other ventral neurons (Ericson et al., 1992). Indeed, the first marker of motor neuron differentiation, Islet-1, is not expressed until stage 15 (Ericson et al., 1992) or about 18–20 hr after neural tube closure and the onset of *dsl-1* expression. Thus, in the period between the initial specification and overt differentiation of neurons, dsl-1 may accumulate to levels that are sufficient to influence neuronal differentiation.

The ability of dsl-1 to inhibit motor neuron differentiation could be involved in preventing the generation of motor neurons and other ventral cell types in the dorsal neural tube. This presupposes that ventralizing signals from the notochord and floor plate can influence dorsal regions of the neural tube. Secreted factors from the floor plate have been shown to diffuse over long distances through the neuroepithelium (Placzek et al., 1990b). Moreover, the position of the ventral boundary of the domain of *dsl-1* expression suggests that signals from the notochord can influence at least two-thirds of the neural tube. Thus, expression of *dsl-1* within the dorsal third of the neural tube could make cells in this region refractory to long-range ventralizing signals from the notochord and floor plate.

The potential contributions of dsl-1 to cell differentiation along the dorsoventral axis of the neural tube will also depend on the range of action of dsl-1 itself. Since dsl-1 is readily secreted from cells in vitro, dsl-1 may diffuse ventrally, beyond the domain of *dsl-1* mRNA expression, to influence the response of cells in intermediate regions of the neural tube. Again, the ability of dsl-1 to antagonize the response of neural cells to ventralizing signals from the notochord and floor plate could be relevant both to the differentiation of motor neurons and to other ventral cell types.

Prevention of *dsl-1* Expression Ventrally May Be Required for Ventral Cell Type Differentiation

Dsl-1 promotes neural crest cell migration and inhibits motor neuron differentiation in the presence of the notochord or floor plate. These findings suggest that the actions of dsl-1 dominate over ventralizing signals. Thus, the inhibition of *dsl-1* expression from ventral regions of the neural tube that is achieved by early signals from the notochord may be necessary for the differentiation of ventral cell types. The absence of ventral cell types in the neural tube of embryos lacking a notochord could, therefore, result either from a ventral expansion in the domain of *dsl-1* expression or from the loss of ventralizing signals. However, in such operated embryos, the neural tube is reduced in size (van Straaten and Hekking, 1991); thus, the death (Homma and Oppenheim, 1992, Soc. Neurosci., abstract) or arrested division (Placzek et al., 1993) of ventral cells could also contribute to the presence of dorsal cell types in regions of the neural tube that appear to be ventral.

Dsl-1 and the TGFβ Family

In addition to *dsl-1*, several other members of the BMP subfamily (also known as the dpp–Vg-related [DVR] subfamily) of TGFβ-like genes are expressed in the embryonic nervous system (Jones et al., 1991). Other BMP-like proteins may therefore mimic the actions of dsl-1 on neural cell differentiation. In preliminary studies, we have found that the induction of motor neurons is also suppressed by COS7 cell–derived BMP-4 (K. B. et al., unpublished data). Expression of the *BMP-4/DVR-4* gene is not detectable in the spinal cord and hindbrain, but the gene is expressed at the ventral midline of the diencephalon (Jones et al., 1991). The expression of *BMP-4* in the ventral diencephalon coincides with and could perhaps contribute to the absence of motor neurons from the embryonic forebrain. The embryonic distribution of most other *BMP* genes is not known, although the *Vg-1*-related gene (*Vgr-1*) (*BMP-6/DVR-6*) is expressed by cells at the dorsal midline of the spinal cord and by cells immediately adjacent to the floor plate (Jones et al., 1991). Of other members of the BMP subfamily whose neural expression pattern has been characterized, growth/differentiation factor 1 (GDF-1) is notable for its widespread distribution (Lee, 1990, 1991). Studies to determine whether GDF-1 mimics the actions of dsl-1 will be important in evaluating the role of this gene family in neural patterning.

The involvement of dsl-1 in the control of cell differentiation along the dorsoventral axis of the neural tube extends the range of activities described for members of the TGFβ family during embryonic development. Studies in Xenopus embryos have provided evidence that activin can control

the identity of mesodermal cell types in a concentration-dependent manner (Ruiz i Altaba and Melton, 1989; Green et al., 1992). In addition, the pattern of expression and possible functions of *dsl-1* in the neural tube have parallels with that of the *dpp* gene in Drosophila embryonic development (Ferguson and Anderson, 1992a, 1992b). Dorsoventral patterning in the early Drosophila embryo involves a dorsal restriction of *dpp* expression (St Johnston and Gelbart, 1987) that is achieved by ventral midline–derived signals that inhibit *dpp* expression ventrally (Ray et al., 1991). Genetic inactivation of this ventral signaling pathway or introduction of *dpp* activity ventrally changes the fate of cells along the dorsoventral axis of the embryo (Ferguson and Anderson, 1992b). In the neural tube, the dorsal restriction of *dsl-1* mRNA by early signals from the notochord could generate a gradient of dsl-1 activity along the dorsoventral axis of the neural tube. Alone or in conjunction with ventralizing signals from the notochord and floor plate, a gradient of dsl-1 could influence the fate of cells according to their dorsoventral position within the neural tube.

Experimental Procedures

RNA Isolation and PCR Amplification

Spinal cord tissue was dissected from 80 E2.5 chicks. Poly(A)$^+$ RNA (20 μg) was isolated from this tissue using an oligo(dT)–cellulose spin column (Pharmacia), and 1.5 μg was used in two first strand cDNA synthesis reactions with either oligo(dT) or random hexanucleotides as primers for the reverse transcriptase reaction. One-third of each of the two cDNA reaction mixtures was combined and used as template for PCR amplification using 100 pmol of the following degenerate primers in a reaction volume of 50 μl: 5′-TGGAATTC TGG(ACG)A(ACGT)-GA(CT)TGGAT(ACT)(AG)T(ACGT)GC-3′ and 5′-GAGGATCCA(AG)(ACGT)GT(CT)TG(ACGT)AC(AGT)AT(ACGT)GC(AG)TG-3′, in which degenerate positions are in parenthesis and restriction sites underlined. These oligonucleotides correspond to the dsl-1 amino acid positions 339–345 and 377–371, respectively. The reaction was cycled twice between 94°C (50 s), 50°C (2 min), and 72°C (2 min), followed by 28 rounds of 94°C (50 s), 55°C (2 min), and 72°C (1.5 min). The reaction products were purified, digested with BamHI and EcoRI, size selected by agarose gel electrophoresis, and cloned into the bacteriophage vector M13mp18. Clones (50) were picked randomly and analyzed on a sequencing gel by comparing their G ladders. One member of each class was sequenced completely.

cDNA Isolation and Sequencing

An E2.5 chick spinal cord cDNA library of 10^6 independent clones was constructed in λZAPII (Stratagene) using 5 μg of the poly(A)$^+$ RNA described above. After amplifying the library, 10^6 clones were screened under standard hybridization conditions, and a ^{32}P-labeled PCR probe was derived from the 116 bp insert of M13 clone B29, representing the *dsl-1* class. Of approximately 25 positive clones, 4 were plaque purified and converted into pBluescript plasmids. Sequence analysis was performed by a combination of primer walking and the subcloning of small restriction fragments into M13. The sequence within and adjacent to the long ORF was determined on both strands by the dideoxy chain termination method (Sanger et al., 1977) using Sequenase (U. S. Biochemicals).

DNA Constructs

The coding region of *dsl-1* was isolated using two PCR primers, ORF-5′ (5′-TGGAATTCATCGATAACGGAAGCTGAAGC-3′) and ORF-3′ (5′-AGCGTCGACATCGATATTCAGCATATACTACC-3′), and cloned into pBluescript SK(−) between the EcoRI and SalI sites. To insert the c-Myc epitope (EQKLISEEDL), two internal primers, each encoding half of the c-Myc and dsl-1 sequences from the epitope insertion site (see Figure 1), were used to produce two PCR fragments, one encoding dsl-1 N-terminal to the insertion site (with primer ORF-5′ and the primer 5′-GCGAATTCGATATCAGCTTCTGCTCTGCTCCTATGCTT-CTCTTGC-3′) and the other encoding the C-terminal region (with primer 5′-CGGAATTCGATATCCGAGGAGGACCTGAACCACTGTC-GGAGAACGTC-3′ and primer ORF-3′). These two fragments were joined using their primer-derived EcoRV sites and cloned the same way as the unmodified coding region. Using nearby primers, this region was sequenced to confirm that no other mutations had been introduced.

A truncated coding region was derived from this construct by cleavage with HindIII, blunting the ends with T4 DNA polymerase, and subsequent religation. This leads to a frameshift mutation that replaces the C-terminal 41 residues of dsl-1 with 9 unrelated ones. The unmodified, the epitope-tagged, and the truncated *dsl-1* coding regions were then cloned into the COS7 cell expression vector pMT21 between the EcoRI and XhoI sites.

In Situ Hybridization Histochemistry

A *dsl-1* cDNA clone was linearized with XbaI (at amino acid position 176) and used to generate a 1 kb [^{35}S]UTP-labeled antisense RNA probe using T7 RNA polymerase. This probe encompasses the 3′ part of the cDNA. Chick embryos were fixed in 4% paraformaldehyde, and 10 μm cryostat sections were mounted on 3-aminopropyltriethoxysilane-treated slides. In situ hybridization was performed essentially as described by Wilkinson et al. (1987) with exposure times ranging from 4 to 10 days. The distribution of *dsl-1* mRNA was confirmed by whole-mount in situ hybridization, performed essentially as described by Harland (1991) using a digoxygenin-11-UTP-labeled RNA probe derived from the template mentioned above (data not shown).

Chick Embryo Manipulations

Notochord grafting and deletion in ovo was performed as described by Yamada et al. (1991). For removal of Hensen's node from stage 9–10 chick embryos in ovo, the embryo was visualized by injection of india ink underneath the cavity between the yolk and embryo. Hensen's node was cut out together with underlying endoderm using fine tungsten needles. After the operation, the window was sealed, and the embryo was incubated for a further 48 hr at 37°C in the humidified incubator. Embryos were then fixed with 4% paraformaldehyde overnight at 4°C and embedded in paraffin for in situ hybridization as described above.

COS7 Cell Transfections

COS7 cells were transfected by the DEAE–dextran method as described by Klar et al. (1992). For small-scale cultures, 60 or 100 mm dishes were used, and conditioned medium was prepared by incubating cells expressing dsl-1 for 48 hr in 3 or 6 ml of OPTI-MEM (Bethesda Research Laboratories), respectively. Large-scale transfections for affinity purification of dsl-1 comprised 15 × 150 mm dishes for transfection with *dsl-1^{myc}* DNA (bearing the Myc epitope) and an equal number of *dpp*- or mock-transfected plates. This yielded 150 ml of dsl-1^{myc}-conditioned medium and 150 ml of COS7-conditioned control medium. The BMP-4 expression plasmid was provided by R. Derynck.

Affinity Purification and Sequence Analysis of Dsl-1^{Myc}

Conditioned medium (50 ml) containing dsl-1^{Myc} was clarified by centrifugation at 30,000 × g and affinity purified on 1 ml of MAb 9E10 (anti-Myc) column (Affi-Gel, Bio-Rad). Dsl-1^{Myc} protein was eluted with 0.1 M glycine–HCl (pH 2.5) and immediately neutralized with 3 M Tris base. The eluate was concentrated and desalted over a 2 ml Centricon-10 microconcentrator (Amicon). The protein concentration of the final fraction (volume approximately 130 μl), as determined by amino acid analysis, was 0.1 μg/ml.

For SDS–polyacrylamide gel electrophoresis, 10 μl of concentrated protein was loaded on a 15% Bio-Rad Mini-Protean II gel under reducing conditions and stained with Coomassie blue. This concentration (60 μl) was used on a preparative gel and blotted onto Immobilon membrane in the absence of glycine. The blot was stained briefly with Coomassie blue, and the major band at 15 kd was excised and subjected to N-terminal protein sequencing on an Applied Biosystems 470A gas phase sequencer–120A phenylthiohydantoin analyzer. The minor protein migrating slightly slower on the gel (at 16.5 kd) was also sequenced and had the identical N-terminus, suggesting that it is an alternately glycosylated form of dsl-1. Affinity-purified conditioned me-

dium from mock-transfected COS7 cells did not contain any detectable protein on a Coomassie blue–stained acrylamide gel.

The concentration of dsl-1^{Myc} used for bioassays was determined on the assumption that all activity resides in the ~15 kd band that represents about 50% of the protein recovered after affinity purification. The total protein in the affinity-purified fraction determined by amino acid analysis was found to be 100 ng/μl, of which 50 ng/μl is assumed to represent active protein. The stock concentration of dsl-1^{Myc} was therefore 3×10^{-6} M. This stock was then diluted 10^5-fold for most assays to give a final condition of 3×10^{-11} M, assuming negligible losses.

Islet-1 Induction Assay

The assay for induction of Islet-1$^+$ cells was carried out as described in detail in Yamada et al. (1993). Intermediate neural plate explants were isolated from HH stage 10 chick embryos (Yamada et al., 1993) and grown in collagen gels alone or with HH stage 10 notochord, HH stage 26 floor plate, or with floor plate–conditioned medium in F12–N3 defined culture medium (Tessier-Lavigne et al., 1988) at 37°C for 48–120 hr. Floor plate–conditioned medium was obtained by culturing 30 HH stage 25–26 floor plate fragments in 1 ml of F12–N3 medium for 48 hr.

After incubation, explants were fixed with 4% paraformaldehyde at 4°C for 1–2 hr, washed with phosphate-buffered saline at 4°C, and gently peeled from the bottom of the dish, and excess collagen gel was trimmed. Explants were incubated with primary antibodies overnight at 4°C with gentle agitation. Rabbit anti-Islet-1 antibodies (Thor et al., 1991; Ericson et al., 1992) and MAb SC1 (Tanaka and Obata, 1984) were used for detection of differentiating motor neurons and MAb 3A10 as a general neuronal marker (Dodd et al., 1988). After washing with phosphate-buffered saline for 2 hr at 22°C, the explants were incubated with Texas red–conjugated goat anti-rabbit antibodies (Molecular Probes) or fluorescein isothiocyanate–conjugated goat anti-mouse immunoglobulin (Boehringer Mannheim) for 1–2 hr. Explants were washed with phosphate-buffered saline at 22°C for 2 hr with at least two changes of buffer and mounted on slides in 50% glycerol with paraphenylene diamine (1 mg/ml). The number of Islet-1$^+$ cells was determined on a Zeiss Axiophot microscope equipped with epifluorescence optics.

Analysis of Neural Crest Differentiation

Intermediate neural plate explants from stage 10 chick embryos were grown in collagen gels as described for analysis of Islet-1 induction. The number of migratory cells was determined by phase-contrast microscopy. Cells were scored as migratory if they were greater than two cell body diameters away from the mass of the intermediate neural plate explant. Identification of surface antigens was performed on cultures fixed with 4% paraformaldehyde using MAb 7412 against chick p75 (Tanaka et al., 1989), MAb HNK-1 (Abo and Balch, 1981), and MAb JG22 (anti-β_1 integrin; Greve and Gottlieb, 1982). For analysis of melanocyte differentiation, intermediate neural plate explants were isolated from HH stage 10 quail (Coturnix coturnix japonica) embryos as described for equivalent chick explants (Yamada et al., 1993) and grown in vitro in collagen gels. Explants were treated with dsl-1^{Myc} (3×10^{-11} M) for 48 hr in F12–N3 medium, at which time the medium was removed and explants washed and placed in F12–N3 medium containing 10% CEE and 10% fetal calf serum for a further 72 hr. Dsl-1 was removed after 48 hr because members of the TGFβ family have been found to inhibit the differentiation of neural crest cells into melanocytes (Stocker et al., 1991; Roger et al., 1992). CEE and serum were added after 48 hr to permit the differentiation of neural crest cells into melanocytes (Baroffio et al., 1988; Maxwell et al., 1988).

Dorsal neural tube and intermediate neural plate explants grown in dsl-1^{Myc} for 48 hr, followed by defined medium lacking CEE or serum for a further 72 hr, gave rise to few (if any) melanocytes. Thus, the presence of CEE and serum appears necessary to support melanocyte differentiation under these conditions. When CEE and serum was included in the medium from the onset of culture, cells migrated from intermediate neural plate explants, and after 120 hr melanocytes were observed.

To prepare CEE, white leghorn chicken eggs were incubated for 11 days at 38°C in a humidified atmosphere. Embryos were removed and homogenized in minimal essential medium by passage through a 30 ml syringe, stirred at 4°C for 1 hr, and then centrifuged for 5 hr at 30,000 × g. The supernatant was collected, filtered, and stored at −80°C until used.

Alkaline Phosphatase Induction in W-20-17 Cells

Induction of alkaline phosphatase activity by dsl-1 was assayed in W-20-17 cells as described (Thies et al., 1992) using recombinant human BMP-2 as a positive control.

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